Biphasic Effect of 1,25-Dihydroxyvitamin D_3 on Human Hair Follicle Growth and Hair Fiber Production in Whole-Organ Cultures

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We have used a whole-organ culture system to investigate the effects of $1,25(OH)_2D_3$ on human hair follicle growth and hair fiber production. Relatively low concentrations (1–10 nM) of $1,25(OH)_2D_3$ stimulated the cumulative growth of hair follicles and hair fibers, by 52% and 36%, respectively (concentration producing 50% of the maximal response [EC_{50}] values of 0.3 nM). The initial rates of follicle and fiber growth were increased, whereas the respective growth periods were unaffected. At higher concentrations of $1,25(OH)_2D_3$, there was a dose-dependent inhibition of both follicle and fiber growth (IC_{50} values of 100 nM), in part due to reduction in the growth periods. There was a marked delay between the onset of $1,25(OH)_2D_3$-induced hair follicle and hair fiber growth inhibition. Incubation of hair follicles with 100 nM $1,25(OH)_2D_3$ resulted in a rapid, transient inhibition of DNA synthesis (55% inhibition at 24 h), followed by a gradual return to control levels at day 4. Prolonged (> 5 h), incubation in the presence of 100 nM of $1,25(OH)_2D_3$ was required for follicle growth inhibition to be manifest. Ro 31-7549, a selective inhibitor of protein kinase C, did not prevent $1,25(OH)_2D_3$-induced inhibition of hair follicle growth. These data suggest that $1,25(OH)_2D_3$ may play a physiologic role in maintaining optimal hair follicle activity, and that elevation of $1,25(OH)_2D_3$ may inhibit hair growth *in vivo. Key words: rickets/alopecia/proliferation. J Invest Dermatol 103:318–322, 1994*

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the principal biologically active metabolite of vitamin D_3 and regulates calcium and phosphate transport in the intestine and mobilization of bone [1]. More recent studies have described the presence of $1,25(OH)_2D_3$ receptors and biologic effects of $1,25(OH)_2D_3$ in tissues not directly related to calcium homeostasis, including parathyroid gland, hematopoietic cells, cancer cells of various origins, pancreatic β cells, and skin (reviewed in [2]). Stumpf *et al* first demonstrated, using thaw-mount autoradiography, that nuclear receptors for $1,25(OH)_2D_3$ (VDR) exist in epidermal keratinocytes, follicular keratinocytes, and sebocytes [3]. In a more detailed autoradiographic study using skin from vitamin D-deficient rats, Stumpf *et al* described the distribution of radiolabeled $1,25(OH)_2D_3$ binding activity in the various cell types that comprise the hair follicle [4]. Nuclear labeling was strongest in cells of the outer root sheath, but labeling was also seen in matrix cells, cortical cells, and all layers of the inner root sheath. In contrast, cells of the dermal papilla and the fibrous sheath were unlabeled. The presence of $1,25(OH)_2D_3$ receptors in hair follicles has been confirmed by immunohistochemistry using an antibody to the intestinal receptor [5]. A possible role for $1,25(OH)_2D_3$ in follicular ontogeny was suggested by the induction of binding activity in the epithelial cells of the hair germ and by the pattern of binding in the developing hair follicle [4].

The finding that $1,25(OH)_2D_3$ exerts potent antiproliferative and prodifferentiative activities on both human [6] and mouse [7] epidermal keratinocytes in culture suggests that $1,25(OH)_2D_3$ may be required for normal epidermal differentiation *in vivo*. In contrast, there have been no studies to date of the effects of $1,25(OH)_2D_3$ on hair follicles *in vitro*, and as a result the role of $1,25(OH)_2D_3$ in regulating hair follicle activity is poorly understood. Indirect evidence for a role for $1,25(OH)_2D_3$ in hair physiology has been obtained from the clinical observation of alopecia in some individuals with type II or calcitriol-resistant rickets [8]. In these patients, circulating levels of $1,25(OH)_2D_3$ are elevated whereas abnormalities in the VDR result in a lack of tissue responsiveness [9]. The relationship between vitamin D resistance and alopecia has been reviewed by Holick [10].

In the present study we have used the human hair follicle whole-organ culture method originally described by Philpott *et al* [11] to investigate the effects of $1,25(OH)_2D_3$ on this tissue. In this culture system, human hair follicles elongate by 1–2 mm over a period of 1–2 weeks, at an initial rate (approximately 0.3 mm/d) comparable to the rate of hair growth *in vivo* in the human scalp [12]. The findings that the linear growth of human hair follicles in organ culture is inhibited by testosterone [13] and by epidermal growth factor (EGF) [11], and that these cultures are capable of the post-translational processing of proteins to produce hair fiber at a physiologic rate [14], argue for the validity of this culture system as a model for the study of the regulation of hair growth. In this report we demonstrate that relatively low concentrations (1–10 nM) of $1,25(OH)_2D_3$ stimulate hair follicle and hair fiber growth, whereas higher concentrations (> 10 nM) are inhibitory, suggesting that this vitamin may have both physiologic and pharmacologic effects on the hair follicle.

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Abbreviation: VDR, vitamin D receptor.

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MATERIALS AND METHODS

Materials 1,25-Dihydroxyvitamin D$_3$(1,25(OH)$_2$D$_3$) was provided by Dr. M. Uskokovic, Hoffmann-La Roche Inc., Nutley, NJ. Ro 31-7549 was provided by Dr. G. Lawton, Roche Products Ltd., Welwyn Garden City, U.K. Hydrocortisone was purchased from Sigma Chemical Co. (St. Louis, MO). Insulin, transferrin, and selenous acid were purchased from Collaborative Research (Bedford, MA). Williams E medium was purchased from Gibco (Grand Island, NY). HEPES, penicillin, and streptomycin were obtained from Halzelton (Lenexa, KS). All other materials were of reagent grade.

Human Hair Follicle Isolation and Culture The method described by Philpott *et al* [11] was used for the isolation and culture of human hair follicles, with minor modifications. In brief, human facial skin was obtained from women undergoing facelift surgery and stored at 4°C until hair follicles were isolated, which always occurred within 36 h of the surgical procedure. Portions of skin were selected in which hair follicles extended 1–2 mm below the dermis-fat junction. The skin was cut with a scalpel into sections of approximately 1-cm^2 area and sectioned transversely at a level immediately below the junction between the dermis and the subcutaneous fat. The subdermal tissue was then submerged in Hanks' BSS/PBS (1:1), and hair follicles were carefully removed from the fat with fine forceps under a dissecting microscope. This procedure removes the lower portion of the hair follicle, including the entire bulb region and part of the hair shaft, and there is little evident damage to the tissue removed. The isolated follicles were incubated at 37°C in 95% air/5% CO_2 in 2-cm^2 wells (2 follicles/well) in 1 ml of Williams E medium containing 10 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The hair follicles were incubated overnight before experiments were initiated. Culture medium was replaced every 3–4 d unless otherwise indicated.

Measurement of the Linear Growth of Hair Follicles and Hair Fibers The method described by Harmon and Nevins [15] was used for the measurement of linear growth of nonpigmented human hair follicles and hair fibers. Hair follicle lengths were determined using an inverted binocular microscope fitted with an eyepiece measuring graticule. The length of the follicle was defined as the distance between the base of the bulb and the cut end of the hair fiber. The length of the translucent, nonpigmented hair fiber was defined as the distance between its base (i.e., the junction with the keratogenous zone) and the cut tip of the fiber. The nonkeratinized length of the hair follicle was defined as the distance between the base of the hair fiber and the base of the hair follicle. Length measurements were taken daily, unless otherwise indicated, until no further significant changes were observed.

Measurement of Rates of DNA Synthesis in Cultured Human Hair Follicles ^{3}H-thymidine was added to the culture medium to a final concentration of 10 μCi/ml, and incubation of the hair follicles was continued for a further 2 h. Unincorporated radioactivity was then removed by incubating the follicles twice in 1 ml phosphate-buffered saline (PBS) for 10 min at 37°C, twice in 1 ml 5% trichloroacetic acid (TCA) for 10 min on ice, and once in 1 ml water for 10 min on ice. Follicles were then transferred with fine forceps to tubes containing 1 ml 0.3 M NaOH and incubated for 18 h at 37°C. After centrifugation for 10 min at 10,000 × *g*, an aliquot was taken for determination of radioactivity by scintillation counting. All cumulative growth and DNA synthesis values are expressed as means ± SD for groups of 8–12 follicles.

Statistical Analysis Mean values and standard deviations were calculated in all experiments. The significance of differences between means were determined using the Student t test.

RESULTS

The effects of varying concentrations of 1,25(OH)$_2$D$_3$ on the timecourse of cumulative growth of cultured human hair follicles and hair fibers are shown in **Fig 1*a,b***, respectively. Growth rates for both hair follicles and hair fibers in untreated cultures were 0.26 mm/d over the initial period of linear growth, after which hair follicle growth declined and ceased at day 10, whereas hair fiber growth continued until day 14. In the presence of 10 nM 1,25(OH)$_2$D$_3$, hair follicles and hair fibers grew at a rate of 0.33 mm/d over the period of linear growth, after which follicle growth ceased at day 10 and fiber growth continued until day 14. Thus, the increase in the total cumulative growth of hair follicles and hair fibers by incubation in 10 nM 1,25(OH)$_2$D$_3$ was due to stimulation of the initial, linear growth phases, rather than to an effect on follicle or fiber growth periods. At higher concentrations (100 nM–10 μM) of 1,25(OH)$_2$D$_3$, there was a dose-dependent reduction in the period of growth of both hair follicles and hair fibers. The rates of follicle and fiber growth over the initial linear period were unaffected by 100 nM 1,25(OH)$_2$D$_3$, but there was a reduction in these rates in the presence of 1 μM 1,25(OH)$_2$D$_3$, whereas incubation in 10 μM 1,25(OH)$_2$D$_3$ resulted in complete arrest of follicle and fiber growth after 1 d of culture. The delay between cessation of follicle and fiber growth was about 4 d in untreated follicles. Whereas this delay was unaffected by incubation with 10 nM and 100 nM 1,25(OH)$_2$D$_3$, it was reduced to 1 d on incubation with 1 μM 1,25(OH)$_2$D$_3$.

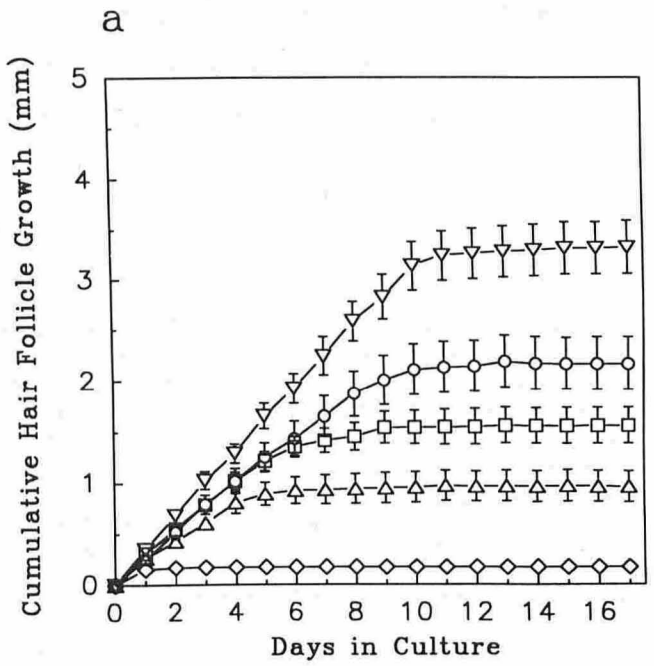
The dose-response relationships for the effects of 1,25(OH)$_2$D$_3$ on the total cumulative growth of hair follicles and hair fibers were biphasic **(Fig 2).** At relatively low concentrations, growth of follicles and fibers was stimulated, to a maximal extent at 10 nM 1,25(OH)$_2$D$_3$ of 52% and 36%, respectively. The concentration producing 50% of the maximal response (EC_{50}) values for both follicle and fiber growth stimulation were 0.3 nM. At higher concentrations, there was a dose-dependent and complete inhibition of follicle and fiber growth, with IC_{50} values of approximately 100 nM in both cases.

We have recently reported that during the course of human hair follicle whole-organ culture, the base of the hair fiber gradually descends towards the follicle base, i.e., the length of the nonkeratinized portion of the hair follicle diminishes, until ultimately hair fiber occupies almost the entire length of the follicle [14]. **Figure 3** shows that the descent of the hair fiber in control cultures began at approximately day 5 and was complete at approximately day 15. A similar timecourse of reduction in nonkeratinized follicle length was obtained for follicles incubated with 10 nM 1,25(OH)$_2$D$_3$. However, in the presence of 100 nM 1,25(OH)$_2$D$_3$, a concentration that reduced follicle and fiber growth periods without affecting initial growth rates, hair fiber descent began at approximately day 2 and was complete on day 11. It should be noted that under all of these incubation conditions the time at which hair fiber descent approached its maximum corresponded closely to the time at which hair fiber production ceased **(Figs 1 and 3).**

To determine the rapidity of the growth-inhibitory response to 1,25(OH)$_2$D$_3$, we incubated hair follicles with 100 nM 1,25(OH)$_2$D$_3$ for varying periods of time and washed and incubated them in control medium. **Figure 4** shows that exposure of hair follicles to 1,25(OH)$_2$D$_3$ for up to 5 h did not significantly affect total cumulative follicle growth, whereas incubation for 12 h or longer resulted in a degree of inhibition comparable to that resulting from continuous incubation in 1,25(OH)$_2$D$_3$. Thus, a relatively prolonged (> 5 h) exposure to 1,25(OH)$_2$D$_3$ was required for its growth-inhibitory effect to be manifest, suggesting that any rapid effects that might be involved in this response either are reversible or are insufficient *per se* to result in growth inhibition.

A timecourse of the effect of 100 nM 1,25(OH)$_2$D$_3$ on rates of whole hair follicle DNA synthesis was undertaken to investigate the possibility that 1,25(OH)$_2$D$_3$-induced inhibition of hair follicle growth resulted from a direct antiproliferative action. **Figure 5** shows that incubation of hair follicles with 100 nM 1,25(OH)$_2$D$_3$ resulted in transient inhibition of follicular DNA synthesis (55% inhibition at 24 h) followed by a gradual recovery to control values at day 4. In contrast, significant inhibition of cumulative follicle growth was only observed after 8 d of treatment of follicles from the same skin specimen, although the growth of 1,25(OH)$_2$D$_3$-treated follicles had essentially ceased by day 6. These data suggest that 1,25(OH)$_2$D$_3$ exerts a rapid antiproliferative effect on follicular keratinocytes, which results in inhibition of follicle growth after a delay of approximately 5 d.

We have used Ro 31-7549, a selective inhibitor of protein kinase C (PKC), to investigate the possible involvement of this enzyme in the growth-inhibitory action of 1,25(OH)$_2$D$_3$ in human hair follicles. **Figure 6** shows that Ro 31-7549 had no effect on the follicular response to 100 nM 1,25(OH)$_2$D$_3$ over a wide range of concentrations of Ro 31-7549 previously shown to block the antiproliferative



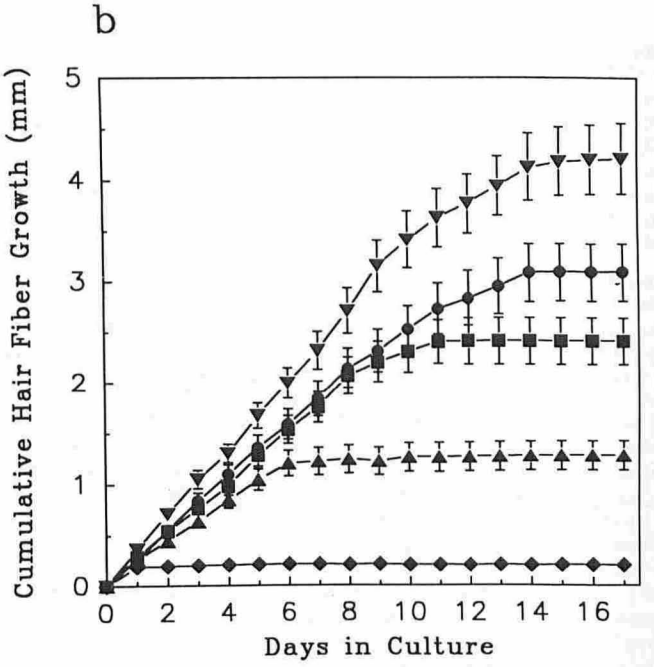


Figure 1. Timecourse of the effect of $1,25(OH)_2D_3$ on the growth of hair follicles *(a)* and human hair fibers *(b)* in whole-organ culture. Human hair follicles were isolated and cultured as described in *Materials and Methods*. Follicles were incubated in the absence (○, ●) and in the continuous presence of $1,25(OH)_2D_3$ at concentrations of 10 nM (▽, ▼), 100 nM (□, ■), 1 μM (△, ▲), and 10 μM (◇, ◆). Length measurements of hair follicles *(a)* and hair fibers *(b)* were made daily, and values are expressed as cumulative growth from day 0 (mean ± SD, N = 8). Similar data were obtained in two additional experiments.

effect of TPA on mouse epidermal keratinocyte cultures [16]. Attempts to block the follicular response to $1,25(OH)_2D_3$ with H7, another selective inhibitor of PKC, were also unsuccessful (data not shown). These findings suggest that the growth-inhibitory response of human hair follicles to $1,25(OH)_2D_3$ does not involve PKC.

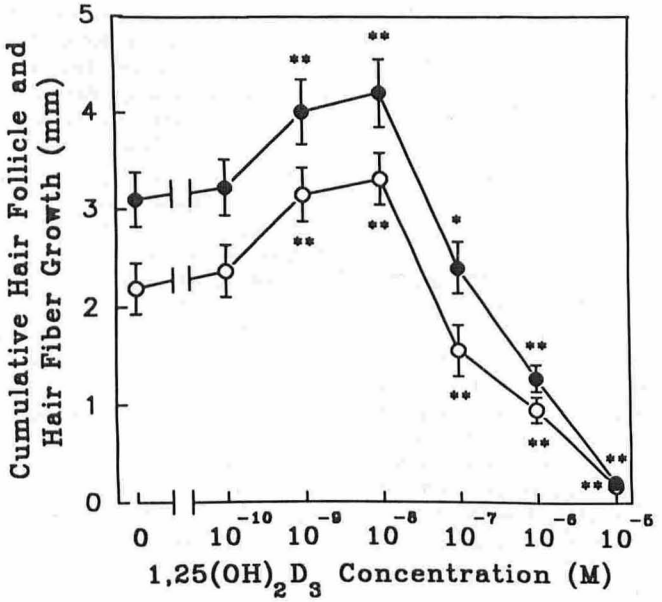


Figure 2. Concentration dependence of the effect of $1,25(OH)_2D_3$ on cumulative growth of human hair follicles and hair fibers in culture. These data were obtained from the experiment described in the legend to **Fig 1**. Values are means ± SD (n = 8) for the cumulative growth of human hair follicles (○) and hair fibers (●) after 17 d of culture, when further growth had ceased. The experiment was repeated twice with similar results. *p < 0.05, **p < 0.01 versus control.

DISCUSSION

Although previous studies have demonstrated the existence of $1,25(OH)_2D_3$ receptors in follicular keratinocytes [4,5], there have been no reports to date on the effects of $1,25(OH)_2D_3$ on hair follicles, either *in vivo* or in tissue culture, and the role of this hormone in the regulation of hair growth remains obscure. Our observation that low levels of $1,25(OH)_2D_3$ (1–10 nM) stimulated rates

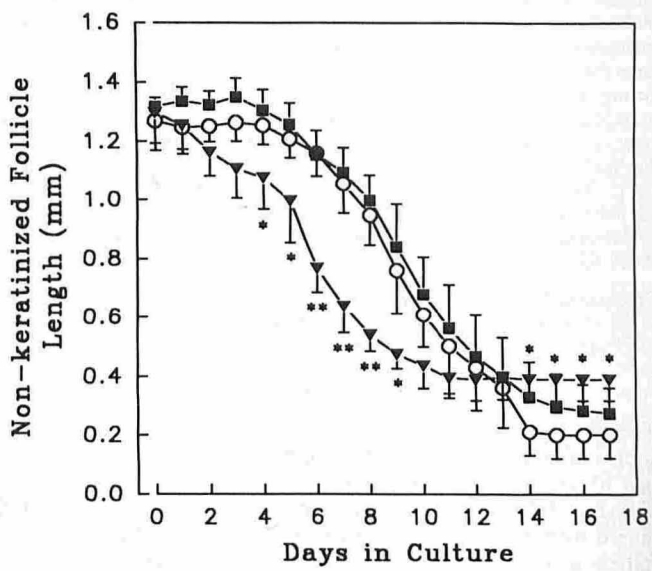


Figure 3. Effect of $1,25(OH)_2D_3$ on the length of the nonkeratinized region of the hair follicle. Human hair follicles were incubated in the absence (○) and in the continuous presence of 10 nM (■) and 100 nM (▼) $1,25(OH)_2D_3$. Measurements were made of the distance between the base of the hair fiber and the base of the hair follicle, as described in *Materials and Methods*. Values represent means ± SD (n = 8). Similar results were obtained in a replicate experiment. *p < 0.05, **p < 0.01 versus control.

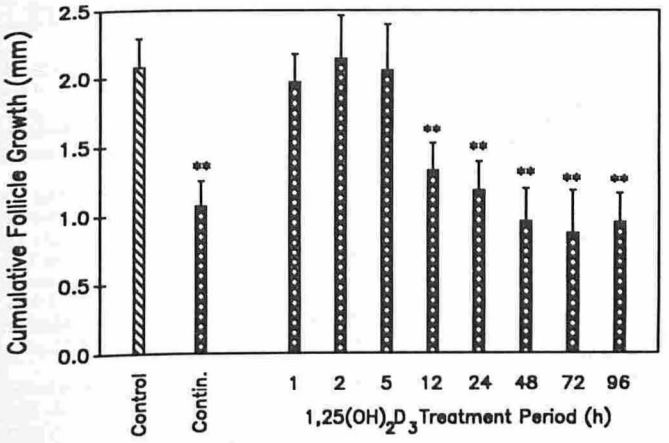


Figure 4. Effect of varying the period of treatment with 1,25(OH)$_2$D$_3$ on the growth of human hair follicles in culture. Human hair follicles were cultured in control medium or in the presence of 100 nM 1,25(OH)$_2$D$_3$, either continuously or for varying periods of time, followed by washing and further incubation in control medium, as indicated. Values are expressed as cumulative hair follicle growth over 11 d of culture (mean ± SD, n = 8), at which time the growth of all follicles had ceased. Similar results were obtained in a replicate experiment. **p < 0.01 versus appropriate control.

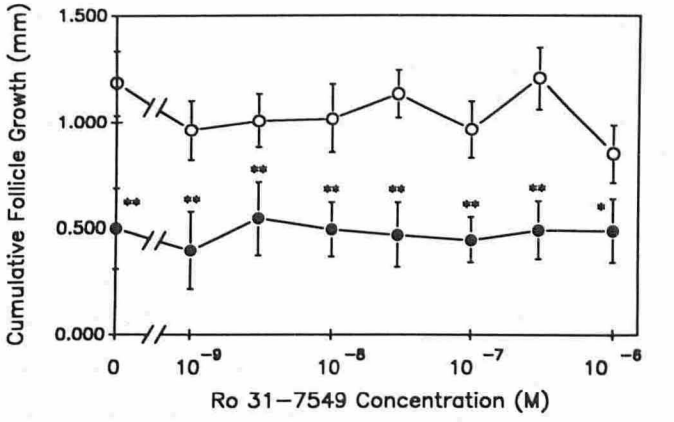


Figure 6. Ro 31-7549, a selective inhibitor of protein kinase C, does not prevent the growth inhibitory effect of 1,25(OH)$_2$D$_3$. Human hair follicles were cultured in the presence of varying concentrations of Ro 31-7549, either in the presence (●) or in the absence (○) of 100 nM 1,25(OH)$_2$D$_3$. Cumulative hair follicle growth (means ± SD, n = 8) was determined after 5 d of culture, as described in *Materials and Methods*. Differences between the growth of follicles pretreated with Ro 31-7549 before addition of 1,25(OH)$_2$D$_3$ and that of follicles treated with 1,25(OH)$_2$D$_3$ alone were not statistically significant. This experiment was repeated with similar results. *p < 0.05, ** < 0.01 versus control follicles treated with the same concentration of Ro 31-7549.

of both linear follicle growth and hair fiber production in human hair follicle cultures suggests that the vitamin may have a role in the maintenance of optimal functioning of mature, recycling hair follicles *in vivo*. However, it is unlikely that mature hair follicles have an absolute requirement for vitamin D, because alopecia is not observed in patients with rickets resulting from dietary vitamin D deficiency [10]. In contrast, a requirement for vitamin D for normal hair follicle development is suggested by the marked alopecia observed in the more severe cases of vitamin D–resistant (type II) rickets [17]. In this condition, an abnormality in the VDR results in the complete abrogation of tissue responsiveness; circulating levels of 1,25(OH)$_2$D$_3$ are normal or elevated, and treatment with calcitriol is ineffective [9,18]. However, some patients with type II rickets have normal hair development, and the relationship between alopecia and vitamin D resistance remains to be fully elucidated [10].

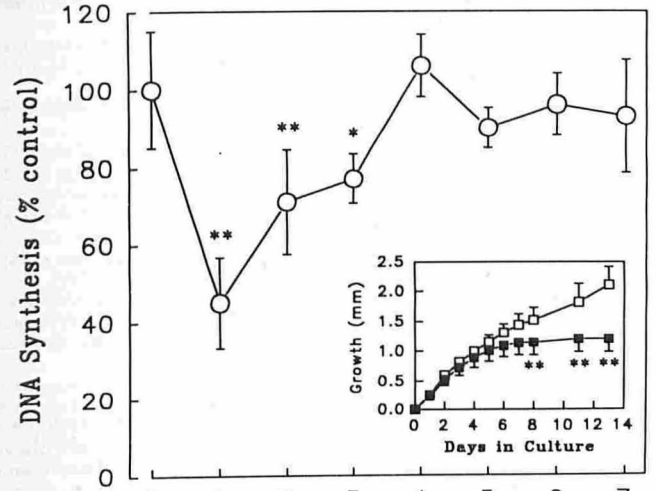


Figure 5. Effect of 1,25(OH)$_2$D$_3$ on DNA synthesis and cumulative growth of cultured human hair follicles. Human hair follicles were isolated and cultured in the continuous presence of 100 nM 1,25(OH)$_2$D$_3$ or in its absence for varying periods of time. Rates of DNA synthesis were determined from the incorporation of ^{3}H-thymidine, as described in *Materials and Methods*. Values are expressed as mean ± SD percentage of control levels (n = 4). *Inset*, timecourse of cumulative hair follicle growth in the absence (□) and continuous presence (■) of 100 nM 1,25(OH)$_2$D$_3$. The hair follicles used for DNA synthesis determination and for cumulative growth measurement were obtained from the same skin specimen. Similar results were obtained in a second experiment. *p < 0.05, **p < 0.01 versus appropriate control.

In the only report to date of enhancement of growth of human hair follicles in organ culture, Taylor *et al* [19] showed that cyclosporin A, which causes hypertrichosis as a common side-effect of its administration, markedly increases the total cumulative growth of human hair follicles in culture. Interestingly, cyclosporin A did not affect the rate of follicle growth over the first 5 d of culture, whereas the period of follicle growth was approximately doubled. In contrast, we show here that 10 nM of 1,25(OH)$_2$D$_3$ increased the rate of follicle growth in its initial, linear phase but did not prolong the growth period. These data suggest that 1,25(OH)$_2$D$_3$ and cyclosporin A stimulate human hair follicle growth by distinct mechanisms.

Taken together, the data presented here suggest that relatively high concentrations of 1,25(OH)$_2$D$_3$ (≥ 100 nM) exert a rapid antiproliferative effect on follicular keratinocytes, which results, after a delay of several days, in inhibition of the linear growth of hair follicles. The existence of VDR in the matrix cells of the follicle bulb [4] is consistent with a direct antiproliferative effect of 1,25(OH)$_2$D$_3$ on this cell population. The finding that 1,25(OH)$_2$D$_3$-induced inhibition of follicular DNA synthesis was both partial and transient (Fig 4) is somewhat unexpected, as 1,25(OH)$_2$D$_3$-induced follicle growth inhibition was monophasic and complete (Fig 1). However, we recently obtained similar results for IL-1–induced inhibition of DNA synthesis and linear growth in cultured human hair follicles [15]. It is possible that 1,25(OH)$_2$D$_3$-responsive and -nonresponsive matrix cell populations exist, the former differentiating to produce hair fiber whereas the latter does not differentiate to produce hair but undergoes compensatory growth following depletion of the pool of 1,25(OH)$_2$D$_3$-responsive cells.

The biologic significance of the antiproliferative effect of higher levels of 1,25(OH)$_2$D$_3$ is unclear. In conditions with elevated circulating levels of 1,25(OH)$_2$D$_3$, such as hyperparathyroidism and chronic granulomatous disorders, there are no abnormalities in hair growth or hair follicle development. Furthermore, patients with renal failure who are given relatively high doses of 1,25(OH)$_2$D$_3$ do

not develop defects in hair growth. However, one might speculate that locally produced $1,25(OH)_2D_3$ plays a role in the induction of catagen, because macrophages accumulate around the hair follicle at this phase of the hair cycle [20] and these cells are capable of the production of $1,25(OH)_2D_3$ from 25-hydroxyvitamin D_3 [21]. An antiproliferative effect of $1,25(OH)_2D_3$ on hair follicles *in vivo* may explain the recent finding that topically applied $1,25(OH)_2D_3$ protects hair follicles from alopecia induced by a variety of chemotherapeutic agents in the neonatal rat [22]. The pharmacologic utility of these agents in cancer chemotherapy derives from their selective toxicity to rapidly proliferating tissues, which also explains their toxic effects on intestinal epithelium, cells of the hematopoietic system, and hair follicles [23]. We speculate that pretreatment with $1,25(OH)_2D_3$ may prevent chemotherapy-induced alopecia by transiently inhibiting follicular keratinocyte proliferation during the period of exposure to the chemotherapeutic agents, thereby protecting follicular matrix cells from cytotoxicity. This hypothesis is supported by the observations that EGF and interleukin-1 both inhibit human hair follicle growth in culture [11,15] and protect against chemotherapy-induced alopecia in the neonatal rat [24,25].

The production of hair fiber by cultured human hair follicles continued unabated in the presence of 100 nM $1,25(OH)_2D_3$ for many days after cessation of follicle growth, and indeed hair growth ceased only as descent of the hair fiber towards the follicle base approached completion **(Figs 1 and 3).** One interpretation of these findings is that $1,25(OH)_2D_3$ does not directly affect the process of terminal differentiation of follicular keratinocytes leading to the production of hair fiber. Rather, the data suggest that inhibition of hair growth following prolonged incubation in 100 nM $1,25(OH)_2D_3$ is a consequence of the almost complete depletion of the population of differentiating follicular keratinocytes that occurs as a result of ongoing terminal differentiation (i.e., hair fiber production) in the absence of matrix cell proliferation.

There is evidence that treatment with $1,25(OH)_2D_3$ results in a rapid activation of PKC in cultured mouse epidermal keratinocytes [26], rat colonic epithelium [27], and chick embryo myoblasts [28]. However, the inability of the selective PKC inhibitor Ro 31-7549 to block $1,25(OH)_2D_3$-induced inhibition of follicle growth (Fig 6) argues against a role for PKC in mediating this response. Ro 31-7549 has previously been shown to inhibit PKC-mediated cellular responses in mouse epidermal keratinocytes [16], human platelets [29], and RBL mast cells [30]. Because the initial PKC-mediated phosphorylation events would be expected to be very rapid, the lack of inhibition of the follicular response to $1,25(OH)_2D_3$ by Ro 31-7549 is consistent with the finding that prolonged exposure to the hormone was required for its inhibitory effects to be manifest **(Fig 4).**

In summary, we show in this report that $1,25(OH)_2D_3$ stimulated human hair follicle growth and hair fiber production at relatively low concentrations, whereas higher concentrations were inhibitory. Our findings are consistent with a role for $1,25(OH)_2D_3$ in the maintenance of optimal hair follicle physiology, and raise the possibility that this hormone may play a role in the regulation of the hair cycle. Further research is required to investigate these possibilities and to identify the cellular targets for $1,25(OH)_2D_3$ within the hair follicle.

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